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19. ABSTRACT

The project reported here focuses on the regulation of neuronal sensitivity by a novel class of protein kinase: an ecto-protein kinase which phosphorylates proteins at the cell surface by extracellular ATP. We proposed that the ecto-protein kinase that our previous studies have described in neuronal cells may play a significant role in the regulation of neuronal signal transduction systems by extracellular ATP, and by extention in mechanisms underlying synaptic plasticity. Our studies are designed to provide experimental evidence in support of this hypothesis. The progress we have made in this project during the period reported here includes the development of novel experimental paradigms for the determination of ecto-protein kinase and its substrates in cultured cells. We used these paradigms in the conclusive identification of the surface phosphoproteins in primary neurons cultured from embryonic brain and in PC12 cloned neuronal cells induced to differentiate by nerve growth factor (NGF). We also determined which of these surface phosphoproteins are regulated by NGF and during neurodevelopment, and which should be at the focus of continued studies on the role of ecto kinase in synaptic plasticity.

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PROGRESS REPORT AND FORECAST

Principal Investigator Yigal H Ehrlich, Ph D
Period of this report July 1, 1989 to March 31, 1990

Concise Summary

The previous Progress Report on this Project covered the period of July 1, 1988 to June 30, 1989 At the request of Ms Linda Tome, we submit here a report for the period indicated above We are providing here a brief background on our research program, a summary of our research objectives, and a description of the accomplishements achieved in our research during this report period

The significant role of extracellular ATP in the regulation of biological functions in a large variety of cells and tissues has been amply demonstrated in a recent Conference of the N.Y. Academy of Sciences dedicated to this issue (Philadelphia, November 1989). ATP and other adenine nucleotides are particularly active in the regulation of neuronal function. It is well documented that neurons store ATP within synaptic vesicles and release it by exocytosis upon stimulation. However, the role of the secreted ATP in the regulation of neuronal function is not fully understood, and the mechanisms underlying this regulation are not completely known. While some of these processes were shown to involve ATP receptors, the Conference mentioned above has emphasized also the importance of investigating the possibility that extracellular enzymes that utilize ATP play an important role in the regulation of neuronal activity by extracellular ATP. In the project reported here this question is addressed by studying the activity of neuronal ecto-protein kinase. This enzyme phosphorylates cell surface proteins by extracellular ATP, and its investigation requires a suitable model system of neurons grown in culture and assayed in an undamaged state. During the period reported here of the operation of our laboratory as part of the CSI/IBR Center for Developmental Neuroscience at Staten Island, we have completed collecting the data-basis on the ecto-protein kinase operating in primary cultures of neurons prepared from the brain of chick embryo. We have identified the proteins at the surface of these cells that serve as

substrates for the ectokinase and become phosphorylated when these cells are incubated with extracellular ATP. After culturing, these cells are maintained in a chemically defined medium and during the subsequent six days they undergo neuronal differentiation. We have also carried out this year a detailed analysis of the developmental pattern of the ecto-protein kinase and its substrates in these cells. The identification of the substrates of ecto-protein kinase in primary neurons was based on the use of new experimental paradigms we have developed during this year to satisfy novel criteria of ecto-kinase activity. These paradigms were developed in experiments carried out with cloned neural cells of the line NG108-15. The same cell line was also used this year in experiments designed to isolate a specific protein substrate of the ectokinase. Another cloned neuronal cell line, called PC12, was used in studies on the potential involvement of ecto-protein kinase in the action of Nerve Growth Factor (NGF). Finally, we have initiated this year experiments intended to identify ecto- phosphoprotein phosphatase activity. To date we have detected significant dephosphorylation of a specific surface protein in another type of cells that secrete ATP: the platelets. These results provided important new guidelines for cotinuation of the research on the role of extracellular protein phosphorylation in neuronal function.

<u>Research Objectives</u>

The specific aims and objectives of the research carried out in this project can be summerized as follows:

(1). Biochemical characterization of the extracellular protein phosphorylation systems operating in primary CNS neurons differentiated in culture. This objective includes experiments demonstrating conclusively the ecto-enzymatic nature of the kinase under investigation.

(2). Isolation and purification of a neuronal ecto-protein kinase and of specific surface proteins that serve as its substrates, and the preparation of antibodies against these components. Emphasis to be placed on obtaining inhibitory antibodies.

(3). Identification and characterization of the surface neuronal proteins whose phosphorylation by extracellular ATP alters during neuronal development and synaptogenesis.

(4). Investigation of the involvement of ecto-protein kinase in the regulation of neuronal function by extracellular ATP, with emphasis on studies of calcium ions mobilization and neuronal plasticity.

Research Accomplishments During this Report Period

A manuscript (in-press) entitled "Ecto-Protein Kinase in the Regulation of Cellular Responses to Extracellular ATP", to be published soon in the Annals of the New York Academy of Sciences, includes descriptions of our recent studies in addition to providing a general background to this research area. Six copies of this manuscript are submitted with this report. Here we describe succinctly our main progress during the period of this report. Progress has been made in five areas:

1. Development of new criteria for conclusive identification of ectokinase and its surface protein substrates.
2. Developmental studies of ecto-protein kinase in primary cultures of CNS neurons.
3. Role of ecto-protein kinase in the action of NGF.
4. Studies of ecto- phosphoprotein phosphatase.
5. Purification of a protein substrate of neural ectokinase.

1. New paradigms for identification of surface phosphoproteins.

Different cell lines and preperations of primary neurons are required for investigating the funtion of various surface phosphoproteins, and this required the development of rigorous experimental paradigms for the identification of these proteins in all cell culture systems. These studies were conducted with a cell line with which we had much previous experience. We have reported that neural cells of the hybrid clone NG108-15 possess an ecto-protein kinase (ePK) that utilizes extracellular ATP to phosphorylate surface proteins (Nature, 320 : 67-70, 1986). In the

present study, these cells were assayed attached to 48-well plates in Krebs-Ringer Hepes buffer. 0.1μM of γ-^{32}P-ATP were used in ePK reactions, and intracellular ATP pools were labeled by incubating the cells with inorganic ^{32}Pi. Incubation of the cells with extracellular AT^{32}P resulted in labeling of about 16 proteins. 67% of maximal labeling occured within 10 min. An excess of non-labeled Pi (1mM added to the medium) had no effect on this phosphorylation. In contrast, addition of the ATP hydrolyzing enzyme apyrase to the medium abolished this phosphorylation. Under the same assay conditions, labeling of proteins in cells incubated with ^{32}Pi was abolished by excess cold Pi, but was not affected by added apyrase. The membrane permeable inhibitor of protein kinase, K-252a, inhibited phosphorylation by both AT^{32}P and ^{32}Pi. The membrane impermeable K-252b inhibited only the phosphorylation by extracellular ATP. Using these criteria we determined that 4 proteins with apparent MW of 170K, 148K, 105K and 13.8K are preferred substrates of ecto-protein kinase in these neural cells. To substantiate this conclusion, we have carried out phosphopeptide mapping of the 105K protein, partially purified from cells labeled by AT^{32}P or ^{32}Pi. Differences in phosphopeptide maps obtained with trypsin, chemotrypsin and V-8 protease verified the existance of phosphorylated sites unique for ecto-protein kinase activity in this protein. These criteria can now be used for the identification of surface proteins in neural cells in which ecto-protein kinase has not been previously studied.

2. Developmental regulation of the phosphorylation of specific surface proteins in primary CNS neurons.

A major emphasis in the research carried out in our lab has been on providing conclusive evidence that CNS neurons have ecto-protein kinase, on detailed characterization of its biochemical properties, and the identification of its cell-surface protein substrates. For this purpose we have implemented the procedures for preparing and maintaining pure primary neuronal cultures from brain tissue. The cells are obtained by dissociation of the telencephalon of 8-day chick embryos. Dissociated cells are plated on poly-L-lysine in the presence of 5% fetal bovine serum which is exchanged 24-36 hours later to a chemically defined medium. During the subsequent six days in culture the plated neurons undergo

substantial neuritogenesis, clustering and maturation. For protein phosphorylation assays with attached cells the neurons are grown in 48- or 96-well plates. Using these procedures we have conducted this year an extensive series of phosphorylation experiments described above as criteria for demonstrating ecto-protein kinase activity. Ecto-protein kinase (ePK) activity was assayed with attached cells incubated in Krebs- Ringer Hepes buffer with 0.1μM of γ-^{32}P-ATP (15 μCi/well). Intracellular proteins were labeled by incubation with equivalent amount of inorganic ^{32}Pi. By comparing the time-course of the phosphorylation of specific protein components with AT^{32}P vs ^{32}Pi, and determining their sensitivity to the addition of excess cold Pi or an ATP hydrolyzing enzyme (apyrase) to the reaction medium, we have identified 8 proteins (M.W.= 115K, 105K, 80K, 64K, 53K, 17K, 13K and 11.7K) that serve as specific substrates for ePK in these primary neurons. Among these, the 11.7K and 13K proteins were phosphorylated only by extracellular ATP and are exclusive substrates of ePK.

During the period of 2 to 6 days in culture the above neurons develop from an immature state of dispersed cells bearing 1-2 short neurites to a neuritic network containing aggregated cells bearing very long and branched neurites that demostrate extensive connectivity and synaptic activity. We have assayed ePK activity at each of the days 2 to 8 in culture, and found that the phosphorylation of the 11.7K and 13K proteins by ePK in the immature, developing neurons is several fold higher than in neurons cultured for 6-8 days. Peak phosphorylation of the 13K protein occured at 3 days in culture. The finding of maximal extracellular phosphorylation of these proteins during neuronal differentiation and synaptogenesis indicates a significant role for ePK in neuronal development. STUDIES OF THESE DEVELOPMENTALLY REGULATED SURFACE PHOSPHOPROTEINS AND THEIR POTENTIAL ROLE IN SYNAPTOGENESIS IS THE MAJOR TARGET OF OUR CONTINUED INVESTIGATION IN THIS PROJECT.

3. Ecto-protein kinase and its substrates in PC12 cells.

Previous studies of the cloned cell line PC12 have enabled major progress in determining the mechanisms of action of NGF. To investigate the role of ecto-protein kinase in the regulation of neuronal differentiation induced by NGF, we have first determined this enzymatic activity in PC12 cells and identified its specific substrates, utilizing all the criteria described above. Briefly, PC12 cells were found to have a very potent ecto-kinase that phosphorylated surface proteins significantly within 10 seconds of incubation with extracellular $AT^{32}P$. Three major substrates were identified in these reactions, proteins of approx. 105K, 40K, and 20K. The phosphorylation of all three was prevented by apyrase, was insensitive to "cold" Pi, and was inhibited by the cell impermeable K-252-b compound, as expected from ePK substrates. The 105K surface protein in PC12 cells co-migrates with the major substrate of ePK in NG108-15 cells, which we are currently isolating (see below). The phosphorylation of the 40K protein by extracellular ATP could take place in buffers containing calcium without magnesium. It may thus be the specific substrate in the regulation of Norepinephrine uptake by ePK, which we have shown to be dependent on calcium, but not on magnesium ions. Most interesting was the phosphorylation by ePK of the 20K protein, which we have found to be stimulated by the addition of 50 ng/ml NGF to the reaction medium (5 min before the NGF was added). In PC12 cells induced to differentiate by 3-days treatment with NGF there was a significant increase in the extracellular phosphorylation of the 20K protein. The role of the 20K protein phosphorylation by ePK in the action of NGF is under current study.

4. Phosphoprotein phosphatase activity at the cell surface.

In parallel studies to those described above, we have found ePK activity in human platelets, and determined that its major substrate is a surface protein with apparent MW of about 40K. The phosphorylation of this protein peaked within 20-30 seconds of the addition of ATP to the medium, followed by almost complete DEphosphorylation within the subsequent 30-60 seconds (manuscript

submitted for publication). This rapid cyclic action of an ePK followed by ecto-phosphatase suggests a potential role for extracellular protein phosphorylation and dephosphorylation in cellular activation processes. Rapid extracellular protein phosphorylation can be measured, as described above, in PC12 cells, which are the target in our current search for ecto-phosphoprotein phosphatase in neuronal cells.

5. Purification of a surface phosphoprotein.

Due to the relatively much larger quantity of protein that can be readily obtained when growing cloned neural cells as compared to primary neurons, we have been develo[ping the procedures for purifying a specific substrate of ePK with cultured NG108-15 cells. In these cells we focus on the surface phosphoprotein characterized best to date--with apparent MW of approx. 105K. It is labeled by incubating intact cells with extracellular AT^{32}P in the presence of excess "cold" Pi, followed by cell harvest and homogenization in buffers containing phosphatase inhibitors (vanadate, fluoride, EDTA & EGTA). In subcellular fractionation by differential centrifugation this phosphoprotein was found in the membrane fraction; from which it could be extracted with 10mM CHAPS. FPLC fractionation of the extract by ion exchange chromatography (Pharmacia Mono Q) and gel filtration (Waters 300SW) provided data consistent with the conclusion that the 105K constitutes part of a membrane-bound macromolecular complex, which may also contain a protein kinase. The next phase of this investigation involves determination of the optimal conditions for the dissociation of this complex and purification of its individual components.